A Functional Dried Fruit Matrix Incorporated with Probiotic Strains: Lactobacillus Plantarum and Lactobacillus Kefir

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Abstract

The consumption of probiotic functional foods, i.e. processed foods enriched with microorganisms that confer health benefits to the host, shows a progressive increase in the last decade due to changes in habits and trends of consumers attracted by the benefits of these products. Currently, the development of fruits and vegetables with probiotic content is a topic of high interest for the probiotic-food consumers as these are a popular item perceived as healthy by consumers, and issues related with lactose intolerance are overcome. The aim of this research study was to develop a new healthy dry food that contains a source of probiotic strains providing some benefits to consumers. Apple was selected as an experimental food matrix and two different probiotic *Lactobacillus* species, *L. plantarum* and *L. kefir*, were tested separately. Samples were taken immediately before and after the drying process in order to determine the viability of bacteria adhered to the matrix. Dried apple cubes were stored in sterile closed glass containers or in sealed bags vacuum packed and normal atmosphere) at room temperature and at 4ºC. The bacterial viability in the dried product was tested at different storage times. For both probiotic strains, a decrease of approximately 2 log cycles in bacterial cell numbers was observed after drying. The bacterial number in apple cubes at the time of storage at room temperature and 4ºC was approximately 1x107 cfu/g. Both probiotic strains died after one month of storage at room temperature, while during storage at 4ºC the cells remained viable after 3 months, with bacterial number around 1x106 cfu/g.

Keywords

Probiotic Bacteria; Storage; Survival; Tray Dryer; Immersion; Vacuum

Introduction

Fruits and vegetables are essential components of the human diet. Apart from being good sources of vitamins, minerals, and fibres, these foods are also a rich source of potentially bioactive compounds (Palafox et al., 2001). Additionally, the consumption of fresh fruits as well as functional foods e.g. probiotics, has increased considerably in recent years, due to the increasing concern in consumers about food and health. Combinations of fruits or vegetables with probiotics (Fito et al., 2001a; Alegre et al., 2010), would create a better, more convenient, product for consumers.

Whilst dairy products are the priority of the development of novel probiotic foods (Puente et al., 2009), lactose intolerance has been reported and an alternative to these dairy products is desirable.

The incorporation of probiotic strains in several food matrixes has been studied maily due to their therapeutical benefits (Lourens-Hattingh et al., 2001; Shah et al., 2010). This represents a challenge, since the viability of the incorporated cells in the food matrix depends on several factors, such as pH, storage temperature, oxygen levels, and presence of competing microorganisms and inhibitors (Mattila-Sandholm et al., 2002). Vacuum impreganation has been reported by several authors as a technique to improve some food characteristics e.g. calcium, iron salts, pH depressors, antimicrobials, etc (Fito and Chiralt, 2000; Fito et al., 2000; Fito et al., 2001b)

Probiotics can be added either to fresh foods with high water activity (aw) or to low aw dry foods. The fresh foods normally have a shelf-life of a few weeks, like yogurts, while the shelf-life of dried products is increased to months, as in the case of milk powder (Weinbreck et al., 2010).

The principal objective of this research work was to create a new healthy dried (non-dairy) food containing a source of probiotic strains bringing some benefits to

consumers namely in the improvement of the immune system. Apple "Golden Delicious" was selected as the food matrix to perform the present research since it is i) easy to handle; ii) relatively cheap; iii) available in many countries at any time of the year; iv) highly nutritious (Obbagy et al., 2009) and v) has a highly porous matrix, allowing entry of the probiotic (Krokida et al., 1998). This apple variety is widely grown and available throughout the year (Anonymous., 2007).

The specific objectives of the present work were: i) to establish the conditions for drying apple in a pilot scale tray dryer; ii) to evaluate the method for incorporating probiotic LAB (i.e. *L. plantarum* and *L. kefir*) in the apple matrix before the drying process, and iii) to evaluate the survival rate of the selected LAB during and after the drying process.

Materials and Methods

Probiotic Bacteria and Growth Conditions

Two Lactobacillus strains, previously described as probiotic strains (Ouwehand et al., 2002; Vinderola et al., 2006; Golowczyc et al., 2007, 2009) were selected for the present study; *L. plantarum*, which can be found in fermented foods, and *L. kefir*, from kefir grains.

These isolates belong to the culture collection of Escola Superior de Biotecnologia, Universidade Católica Portuguesa. Both strains were stored in MRS broth (Pronadisa, Spain, Madrid) plus glycerol (30% v/v) at -80ºC, until use. To prepare the pre-inoculum, a sterile tube with MRS broth (15 mL) was inoculated with a pure colony of the selected strain. This suspension was incubated at 37°C for 24 hours. The pre-inoculum (5 mL, 1% v/v) was added to 500 mL of MRS broth and incubated at 37°C for 24 hours. This culture was centrifuged in sterile 50 mL Falcon tubes, (5000 x g at 4°C; Hettich Zentrifugen Rotina 35R, Germany) for 5 minutes followed by two washes of the pelleted cells by re-suspension and centrifugation, with sterile Ringer's solution (Merck, Germany, Darmstadt), under the same conditions. Cell pellets were then re-suspended in 20 mL of Ringer's solution in each Falcon tube in order to concentrate the probiotic suspension before addition to the fruit matrix.

Sample Preparation

The apples used in this study were the variety 'Golden Delicious' obtained in local markets from the region of Porto. This variety was chosen because of its sweet flavour, and the pulp is smooth with a crunchy texture (Molin, 2001) and as well it does not oxidize very easily during processing. Apples were washed with water, peeled and cut into cubes of about 2 cm sides, using a mold to obtain cubes with the same size and shape. These apple cubes were immediately immersed in sterile Ringer's solution to inhibit the oxidation of the matrix until submersion in the concentrated cell suspension prepared as described above (500 g of apple to 1L of sterile Ringer's solution). Cubes immersed in Ringer's solution were recovered by filtration using sterile gauze.

Adherence of Probiotic Cells

To promote adherence of probiotic cells into the apple matrix, two techniques were tested: immersion and vacuum impregnation (Betoret et al., 2003).

1) Immersion

In the immersion technique, the apple cubes were immersed in the probiotic suspension for one hour. In order to make this adherence uniform in all cubes, a gentle agitation over time was applied, ensuring that all cubes were immersed in the solution under the same conditions. Afterwards, the cubes were recovered by filtration under aseptic conditions using sterile gauze, placed on trays and then into the dryer (UOP8, Armfield, United Kingdom).

2) Vacuum

A vacuum impregnation technique was also tested (Maguiña et al., 2002). Apple cubes were immersed in the concentrated cellular suspension, in a bag suitable for vacuum sealing. A pressure of 50 mbar for 1.2 seconds was established, with subsequent sealing of the bag (Multivac A300/52 Vacuum, United States of America). The bags were opened and the cubes were removed, also using sterile gauze, placed on trays that were loaded into the dryer, where the drying would be accomplished. Before and after addition to apple cubes, enumeration of LAB was performed as described below.

Drying Conditions

A pilot-scale tray dryer (which allows the drying of wet solid products by flowing hot air over the trays) was used. Two different temperatures and two different air velocities were tested. Initially, drying was performed at room temperature (ca. 20ºC) and

with an average speed of air circulation of 0.5 m/s. These conditions were used to check if the probiotic bacteria, adhered to apple cubes by the immersion technique used for the sample preparation, suffered any decrease in viability, during dehydration. The duration of this experiment was one week and during this time several samples were taken and survivors enumerated. Subsequently, these conditions were changed. The temperature and speed of air flow were increased to 40ºC and 1.5 m/s, respectively. In this second experiment, drying was faster essentially due to the increase in temperature. Drying of apple cubes with adhered LAB, by immersion at normal pressure or vacuum techniques, occurred in approximately, 27 to 30 hours. Samples were taken during drying process and survivors were enumerated. The relative humidity (RH) of the drying air and the aw of the apple cubes during the drying process were measured in order to determine the effect of the RH on the drying of the cubes (Himmelfarb et al., 1962).

Storage Conditions

After drying at 40ºC, the samples were divided into two groups, one to test the effect of atmospheric conditions, and the other to test the effect of temperature on the viability of the adhered probiotic bacteria. A portion of the dried apple was stored under vacuum conditions and the other in sterile Schott flasks (full flasks with almost no head space) under normal atmosphere conditions. Apple cubes that were submitted to vacuum conditions (Multivac A300/52 Vacuum, United States of America) were divided into bags and sealed after a pressure of 1 mBar was established. These two groups of samples (vacuum packed and normal atmosphere) were stored and then divided in order to determine if storage temperature had any effect on cell viability. Two storage temperatures were tested: room temperature (ca. 20°C) and 4°C.

Bacterial Enumeration

One gram of apples (freshly inoculated) were added to 9 mL of sterile Ringer's solution and mixed in the stomacher (BagMixer® 400 P, Interscience, France) for one minute. Then, serial decimal dilutions were performed and LAB were enumerated by the drop count technique on MRS agar (Biokar, France, Beauvais) plates. Colony counting was performed after incubation at 37ºC for 24 hours. The same procedure was followed for samples after drying but the sample weight was 5 g added to 45 mL of sterile Ringer's solution.

Results and Discussion

The main objective of the present research was to create a dry fruit matrix with a high number of viable probiotic cells (at least > 1x107 cfu/g); therefore it was crucial: i) to obtain an initial suspension in which the matrix would be immersed, with a high cell concentration (~1010 cfu/mL); ii) to assure a high adherence of the probiotics to the fruit matrix; iii) to guarantee that after drying, the viability of adhered cells was still high.

A concentrated probiotic suspension (ca. 1010 cfu/mL) was produced to allow a high incorporation of the cells into the food matrix by the two different techniques, immersion and vacuum. It was established that one hour of contact would be sufficient to promote good adherence with a high concentration of viable cells (ca. 109 cfu/mL; data not shown).

In terms of cell numbers, after one hour immersion, a difference of one log cycle approximately, was observed between the initial probiotic suspension and the immersed apple matrix. According to Fito et al. (2001a), a vacuum technique would allow the introduction of controlled quantities of a solution into the porous structure of fruits. In fact, these authors described that vaccum impregnation could introduce into the fruit and vegetables, controlled quantities of a given solution. However, in the present study, no differences have been observed between the two tested techniques, since the same concentration of viable cells in the apple matrix was achieved at the same cell suspension concentration by both techniques (data not shown).

Apple cubes with adhered probiotic bacteria were dried. After drying, it was observed that cubes that had been subjected to immersion under vacuum to promote adherence, presented lower viable numbers than apple cubes that had been subjected to immersion at normal pressure conditions (Figs. 1 and 2 for *L. kefir* and *L. plantarum*, respectively). In the course of comparison of both techniques, a reduction of approximately 2 log cycles was observed for both strains for apple samples that were just immersed, whereas losses were approximately of 4 log cycles for *L. plantarum* and near 3 log cycles for *L. kefir* for apple cubes that were vacuum treated. Therefore, the vacuum technique did not confer any advantages in either increasing the numbers of cells adhered or in

stability during drying. This was also confirmed in the study by Alzamora et al. (2005), also using apple cubes as a food matrix and a different range of vacuum pressures.

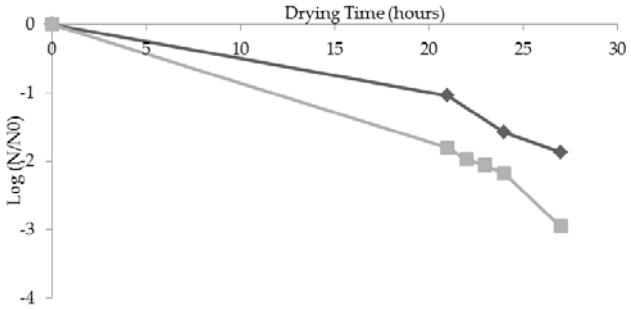

FIG. 1. VIABILITY OF *LACTOBACILLUS KEFIR* CELLS IN APPLE CUBES AFTER ADHESION; BY THE IMMERSION TECHNIQUE: BY THE VACUUM TECHNIQUE. ERROR BARS INDICATE NO VARIABILITY BETWEEN ASSAYS.

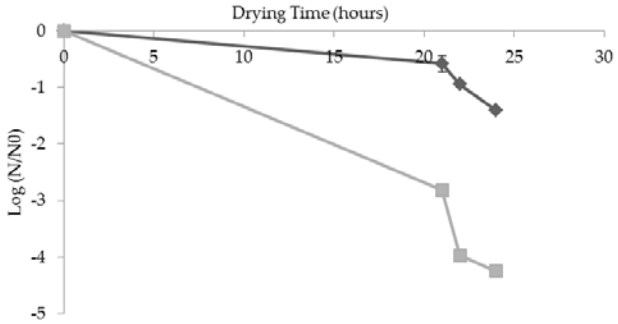

FIG.2. VIABILITY OF *LACTOBACILLUS PLANTARUM* CELLS IN APPLE CUBES AFTER ADHESION; BY THE IMMERSION TECHNIQUE; BY THE VACUUM TECHNIQUE. ERROR BARS INDICATE NO VARIABILITY BETWEEN ASSAYS.

It was also noted that vacuum immersed samples had a worse visual aspect after drying, with more damage observed when compared with samples that were not subjected to vacuum.

Even with the reduction of viable cells (≤2 log cfu/g) observed in the apple cubes inoculated by simple immersion, the lactobacilli continued to be present in large numbers, (ca. 107 cfu/g), even at the end of the drying process. Much greater reductions of viable cells (ca. 3-4 log cfu/g) were noted for both lactobacilli in apple cubes inoculated by vacuum immersion (Figs. 1 and 2).

When these results were compared with Betoret et al. (2003), some differences in the final concentrations of incorporated cells in the final product were observed. To promote adherence (Betoret et al., 2003), fruit juices or even milk inoculated with probiotic bacteria were used. These suspensions were then put in contact with the apple slices to promote the incorporation of the bacteria in the matrix. This step led to a better incorporation of the probiotic bacteria into the apple slices, when compared to the results obtained in the current study. The use of juice or milk seemed to confer some protection to the probiotic cells, making them more resistant to drying. The pressure used for vacuum immersion was the same, but Betoret et al. (2003) applied it for a longer period. This may have had some advantages for the incorporation of the cells, since in the currently reported study, vacuum was applied for only 1.2 seconds at 50 mBar instead the 10 minutes used by Betoret et al. (2003) to promote adherence. These differences in time could lead to different adherences of the cells to the matrix.

Dried apple cubes incorporated with the two probiotic LAB by both methods, were stored at room temperature (ca. 20°C) in closed glass bottles in the dark for up to 25 days. After 24 days of storage, viable cells of *L. kefir*, incorporated by either method, had decreased by ca. 1 log cfu/g (Fig.3).

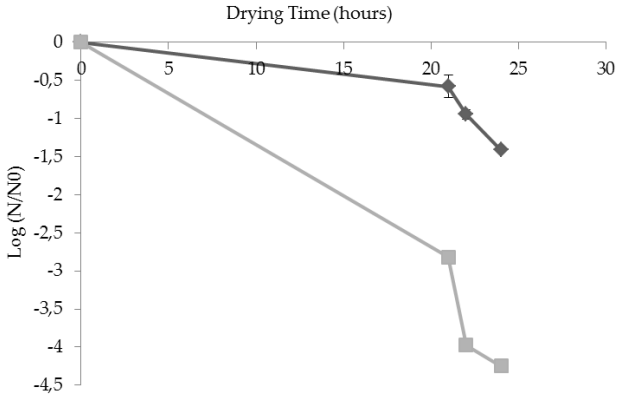

FIG.3. VIABILITY OF *LACTOBACILLUS PLANTARUM* CELLS IN APPLE CUBES DURING DRYING TIME;. IMMERSION TECHNIQUE TO PROMOTE ADHERENCE; VACUUM TECHNIQUE TO PROMOTE ADHERENCE. ERROR BARS INDICATE NO VARIABILITY BETWEEN ASSAYS.

After 20 days of storage, *L. plantarum* viable cells incorporated into apple by immersion, had decreased by ca. 0.5 log cfu/g, but cells incorporated by the vacuum technique had decreased by ca. 1.5 log cfu/g (Fig.4). In an attempt to minimize the loss of viability of *L. plantarum*, vacuum infused apple cubes were also stored under vacuum, since it was possible that air – oxygen — was deleterious for cell survival. After just eight days of storage at ambient temperature, vacuum-stored cells had lost ca. 4 log cfu/g, and could not be recovered thereafter; cells could be recovered after 25 days storage in normal atmosphere, although with a

loss of viability of ca. 2 log cfu/g (Fig. 5).

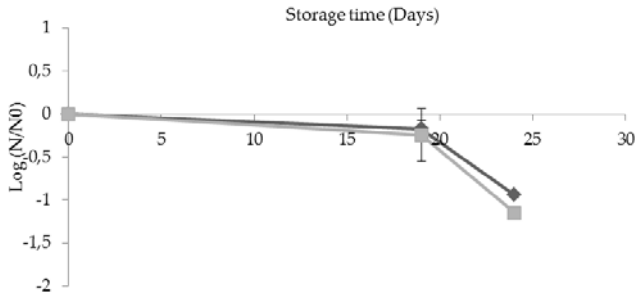

FIG.4. VIABILITY OF LACTOBACILLUS KEFIR CELLS IN APPLE CUBES DURING DRYING TIME;. IMMERSION TECHNIQUE TO PROMOTE ADHERENCE; VACUUM TECHNIQUE TO PROMOTE ADHERENCE. ERROR BARS INDICATE NO VARIABILITY BETWEEN ASSAYS.

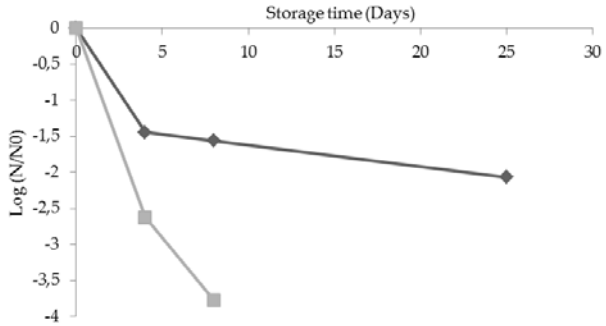

FIG.5. EFFECT OF VACUUM TECHNIQUE USED TO PROMOTE ADHESION OF LACTOBACILLUS PLANTARUM, ON ITS SURVIVAL DURING STORAGE AT ROOM TEMPERATURE. STORAGE AT NORMAL ATMOSPHERE; STORAGE UNDER VACUUM.

Over several replicated experiments with both LAB incorporated into apple cubes by immersion, and drying by air at 40°C, the cell viability losses by drying, were between 1.5 and ca 3 log cfu/g (data not shown). A possible reason for the differences recorded is that the RH of the heated air was not controlled, thus giving different rates of drying, and with low RH there may have been rapid surface dehydration and prolonged dehydration of the interior.

Golowczyc et al., (2009) observed, using the same strains used in this study, that *L. plantarum* was more resistant to high temperatures than *L. kefir*. As reported by other authors, these strains of *Lactobacillus* are capable of growth in this range of temperature (De Vos, 2009), so cell death is probably the decrease in water content, leading to shrinkage of the cell membrane and to the death of cells.

After one month of storage at room temperature, the viability of cells in apple slices decreased by 4 log cycles. However, storage at 4°C resulted in a loss of viability of only 1 log cfu/g even after 65 days of storage (Fig. 6). As in the study of Alzamora et. al. (2005), samples that were incorporated with probiotics, only lost one log cycle of viability during storage at 4 ºC and could remain stable for long periods at that temperature. So, it was possible to conclude that, for the conditions investigated, storage in normal atmosphere at 4ºC was the best way to preserve probiotic cell viability in dried apple cubes.

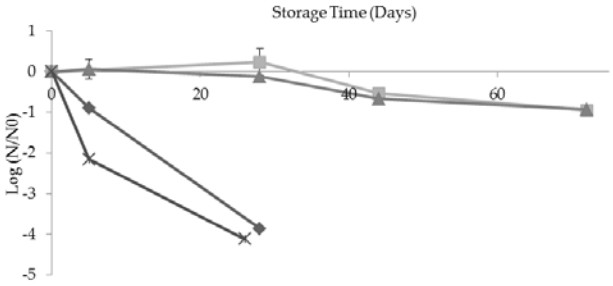

FIG.6. SURVIVAL OF CELLS IN DRIED APPLE CUBES DURING STORAGE. LACTOBACILLUS PLANTARUM SURVIVAL AT ROOM TEMPERATURE; LACTOBACILLUS PLANTARUM SURVIVAL AT 4 ºC; LACTOBACILLUS KEFIR SURVIVAL AT ROOM TEMPERATURE; LACTOBACILLUS KEFIR SURVIVAL AT 4 ºC.

After drying the apple matrix, the cubes were stored for at least one month, to check the cell viability and shelf life under storage conditions. Several factors could influence the quality of the product, including temperature, moisture content, and atmosphere composition in which the product is stored (Anonymous, 2001).

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